# Peroxisome-proliferator-activated receptor $\alpha$ agonists inhibit cyclo-oxygenase 2 and vascular endothelial growth factor transcriptional activation in human colorectal carcinoma cells via inhibition of activator protein-1

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Recent evidence indicates that PPAR (peroxisome-proliferator-activated receptor) $\alpha$ ligands possess anti-inflammatory and anti-tumoural properties owing to their inhibitory effects on the expression of genes that are involved in the inflammatory response. However, the precise molecular mechanisms underlying these effects are poorly understood. In the present study, we show that tumour promoter PMA-mediated induction of genes that are significantly associated with inflammation, tumour growth and metastasis, such as COX-2 (cyclo-oxygenase 2) and VEGF (vascular endothelial growth factor), is inhibited by PPAR$\alpha$ ligands in the human colorectal carcinoma cell line SW620. PPAR$\alpha$ activators LY-171883 and WY-14,643 were able to diminish transcriptional induction of COX-2 and VEGF by inhibiting AP-1 (activator protein-1)-mediated transcriptional activation induced by PMA or by c-Jun overexpression. The actions of these ligands on AP-1 activation and COX-2 and VEGF transcriptional induction were found to be dependent on PPAR$\alpha$ expression. Our studies demonstrate the existence of a negative cross-talk between the PPAR$\alpha$- and AP-1-dependent signalling pathways in these cells. PPAR$\alpha$ interfered with at least two steps within the pathway leading to AP-1 activation. First, PPAR$\alpha$ activation impaired AP-1 binding to a consensus DNA sequence. Secondly, PPAR$\alpha$ ligands inhibited c-Jun transactivating activity. Taken together, these findings provide new insight into the anti-inflammatory and anti-tumoural properties of PPAR$\alpha$ activation, through the inhibition of the induction of AP-1-dependent genes that are involved in inflammation and tumour progression.

Key words: cyclo-oxygenase 2 (COX-2), LY-171883, peroxisome-proliferator-activated receptor $\alpha$ (PPAR$\alpha$), SW620 cell, vascular endothelial growth factor (VEGF), WY-14,643.

## INTRODUCTION

PPARs (peroxisome-proliferator-activated receptors) are members of the nuclear receptor family of transcription factors, a diverse group of proteins that mediate ligand-dependent transcriptional activation and repression. They modulate gene transcription in response to specific ligands by binding as heterodimers with the RXR (retinoid X receptor) to specific PPREs (peroxisome-proliferator-response elements) on target genes (reviewed in [1]). So far, three distinct forms of PPARs have been described, named PPAR$\alpha$, $\beta$ (also called $\delta$) and $\gamma$, each encoded by a different gene and showing a distinct tissue distribution [2]. In humans, PPAR$\alpha$ is expressed in intestine, skeletal muscle, liver, kidney, adipose tissue and vascular endothelial cells [3,4]. Several peroxisome proliferators have been shown to bind PPAR$\alpha$ and to regulate transcriptional activity of target genes. These include the fibrate class of hypolipidaemic drugs, NSAIDs (non-steroidal anti-inflammatory drugs), fatty acids and eicosanoids (reviewed in [5]).

Interest in PPARs has increased dramatically since they were found to be involved in the regulation of processes as diverse as lipid and glucose metabolism, cell growth and inflammation. Therefore, in addition to their well known effects in diabetes, pharmacological agents that target PPARs may have therapeutic applications in cancer and inflammatory diseases [6,7]. In this sense, several reports have shown the potentially beneficial chemopreventive effect of PPAR$\alpha$ ligands in colon carcinogenesis [8–11]. Many of the anti-inflammatory and anti-neoplastic properties of PPAR ligands are due to their inhibitory effects on gene transcription [6,12]. PPAR$\alpha$ agonists are involved in the transcriptional repression of a variety of inflammatory genes [13–15]. These effects seem to be mediated by the inhibition of various transcription factors such as NF-$\kappa$B (nuclear factor-$\kappa$B), AP-1 (activator protein-1) [16] and specificity protein 1 (Sp-1) [17].

On the other hand, a growing body of evidence has highlighted the contribution of COX-2 (cyclo-oxygenase 2) and VEGF (vascular endothelial growth factor) genes in inflammation, tumour growth and angiogenesis (reviewed in [18,19]). COX-1 and COX-2 catalyse the conversion of AA (arachidonic acid) into $PGH_2$ (prostaglandin $H_2$), the key step in the biosynthesis of prostanoids. COX-1 is constitutively expressed in most tissues, whereas COX-2 expression is induced by cytokines, mitogens and tumour promoters in a discrete number of cell types (reviewed in [20]). COX-2 is aberrantly overexpressed in many human cancers, most notably of colon origin [21], being considered to play an essential role in cancer progression, especially in colon carcinoma. Multiple studies have revealed a role of selective COX-2 inhibitors in decreasing the risk of developing colon cancer and

Abbreviations used: AP-1, activator protein-1; Apc, adenomatous polyposis coli; COX, cyclo-oxygenase; DBD, DNA-binding domain; EMSA, electrophoretic mobility-shift assay; FBS, foetal bovine serum; Ion, A23187 calcium ionophore; JNK, c-Jun N-terminal kinase; MEM, minimal essential medium; NF-$\kappa$B, nuclear factor $\kappa$B; NSAID, non-steroidal anti-inflammatory drug; 15d-$PGJ_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$; PPAR, peroxisome-proliferator-activated receptor; PPRE, peroxisome-proliferator-response element; RLU, relative luciferase units; RT, reverse transcription; VEGF, vascular endothelial growth factor.

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in suppressing tumour formation and growth in animal models [18,22]. Besides, accumulating evidence supports a key role for VEGF in cancer, contributing to tumour neovascularization and dissemination. Increased expression of this factor has been found in most tumours, and blockade of VEGF expression or activity ameliorates tumour growth *in vivo* (reviewed in [23,24]).

In the present study, we have tested the effect of two structurally different PPAR$\alpha$ activators as the hypolipidaemic drug WY-14,643 and the leukotriene $D_4$ antagonist LY-171883 in the regulation of COX-2 and VEGF gene expression in the colon carcinoma cell line SW620. Our results show that PPAR$\alpha$ activators specifically suppressed transcriptional induction of COX-2 and VEGF by phorbol esters. These drugs inhibited up-regulation of COX-2 and VEGF by inhibiting AP-1-mediated transcriptional activation through a negative cross-talk between PPAR$\alpha$ and AP-1 transcription factors.

## EXPERIMENTAL

### Reagents

Opti-MEM®, RPMI 1640, MEM (minimal essential medium), glutamine and antibiotics were from Invitrogen. FBS (foetal bovine serum) was purchased from Euroclone. PMA and Ion (calcium ionophore A23187) were from Sigma. The PPAR$\alpha$ agonists LY-171883 and WY-14,643, the cyclopentenone 15d-$PGJ_2$ (15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$) and the anti-COX-2 monoclonal antibody were from Cayman Chemical. Horseradish-peroxidase-coupled anti-mouse antibodies and the SuperSignal enhanced chemiluminescence detection system were from Pierce. Oligonucleotides were synthesized by Invitrogen. [$^{32}$P]ATP for radioactive labelling was from Amersham Biosciences. Reagents for DNA transfection and luciferase assays were from Promega. The most commonly used chemicals were from Sigma and Merck.

### Cell culture

The human colon carcinoma cell line SW620 was grown in RPMI 1640 medium supplemented with 10 % FBS, 2 mM L-glutamine and antibiotics. The human colon carcinoma cell line Caco-2 was grown in MEM supplemented with 10 % FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids and antibiotics. The COS-7 cell line was cultured under standard conditions in DMEM (Dulbecco's modified Eagle's medium). Cells were grown and maintained at 37 °C in a humidified atmosphere containing 5 % $CO_2$ up to 70 % confluence and trypsinized with 0.25 % trypsin and 2 mM EDTA for experimental use. Cells were changed to medium with 0.5 % FBS before treatment with pharmacological reagents. No evidence of significant toxicity was observed at the doses used in any of our experiments as determined by the WST-1 cell viability assay.

### Plasmid constructs

The COX-2 promoter construct COX-2-LUC contains the $-1796$ to $+104$ region of the human COX-2 gene in the pXP2LUC plasmid. The 431-COX-2-LUC mutant was generated by site-directed mutagenesis using the oligonucleotide 5′-GACAGGAGAGTG-GtacCTACCCCCTCTGCTCCC-3′ (nucleotides $-236$ to $-204$ of the human COX-2 gene containing the NF-$\kappa$B site as described previously [25]). Lower-case letters indicate mutated positions. The VEGF-LUC plasmid contains the region $-1910$ to $+379$ of the human VEGF promoter [26]. The $-73$Col-LUC plasmid includes the AP-1-dependent region (from $-73$ to $+63$) of the human collagenase promoter fused to the luciferase gene [27]. The NF-$\kappa$B-Luc reporter plasmid contains a three tandem repeat of the NF-$\kappa$B-binding motif of the H-2k gene upstream of the thymidine kinase minimal promoter [28]. The expression plasmid encoding PPAR$\alpha$ was a gift from Dr B. Staels (Departement d'Atherosclerose, Institut Pasteur de Lille et Université de Lille, Lille, France) [13]. The PPAR$\gamma$ expression vector and those containing the transactivation ligand-binding domains of PPAR$\alpha$ or PPAR$\gamma$ fused to the GAL4 DBD (DNA-binding domain) (pCMX-Gal-L-mPPAR$\alpha$) were provided by Dr R. M. Evans (Howard Hughes Medical Institute, The Salk Institute for Biological Sciences, San Diego, CA, U.S.A.) [29]. The reporter plasmid PPRE-LUC containing three copies of the PPRE of the ACO (acyl-CoA oxidase) was a gift from Dr B. Belandia (Instituto de Investigaciones Biomédicas Alberto Sols CSIC-UAM, Madrid, Spain). The expression plasmid encoding human p65 was a gift from Dr J. Alcamí (Centro Nacional de Microbiologia, ISCIII, Majadahonda, Madrid, Spain). The expression plasmid pRSV-c-Jun has been described previously [30]. The GAL4-c-Jun plasmid expressing the first 166 amino acids of the human c-Jun fused to the DBD of the yeast GAL4 transcription factor (amino acids 1–147) was obtained from Dr P. Angel (Division of Signal Transduction and Growth Control, Deutsches Krebsforschungszentum, Heidelberg, Germany). The GAL4-LUC reporter plasmid contains five copies of the GAL4 DBDs fused to the luciferase gene.

### mRNA analysis

Total RNA was obtained from SW620 or Caco-2 cells by using the TRIzol® reagent (Invitrogen) and analysed by quantitative real-time RT (reverse transcription)–PCR analysis. RT of total RNA was performed using the components of the High Capacity cDNA Archive kit (Applied Biosystems), and amplification of the COX-2 mRNA was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI PRISM 7900HT instrument (Applied Biosystems). All samples were run in triplicate. COX-2 mRNA, VEGF mRNA, 18 S rRNA-specific primers and TaqMan MGB probes were from Applied Biosystems. Relative quantification of gene expression by real-time RT–PCR was calculated by the comparative threshold cycle ($\Delta\Delta C_T$) method using the manufacturer's software and instructions. Data were normalized to the endogenous control 18 S rRNA to account for variability in the initial concentration of RNA and in the conversion efficiency of the RT reaction.

### Immunoblot analysis

SW620 or Caco-2 cells were disrupted in ice-cold lysis buffer (50 mM Tris/HCl, pH 8, 10 mM EDTA, 50 mM NaCl, 1 % Nonidet P40, 0.1 % SDS, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin and 1 mM PMSF). Solubilized extracts (30 $\mu$g) were separated by SDS/PAGE on 10 % polyacrylamide gels, and transferred on to nitrocellulose filters. The membranes were incubated overnight at 4 °C with mouse anti-COX-2 monoclonal antibody (1:1000 dilution) in blocking buffer. The filters were washed and incubated with rabbit anti-mouse IgG secondary antibody linked to horseradish peroxidase. The stained bands were visualized using the SuperSignal enhanced chemiluminescence detection system.

### Transfection and luciferase assays

SW620 or COS-7 cells were transiently transfected by the Lipofectamine™ PLUS reagent as recommended by the manufacturer (Invitrogen). Exponential growing cells were incubated for 4 h at 37 °C with a mixture of the correspondent reporter plasmid, Lipofectamine™ PLUS reagent in OptiMEM®. In co-transfection experiments, different quantities of the correspondent expression plasmids were included as described in the Figure legends. The

total amount of transfected DNA was kept constant by using empty expression vectors. For transactivation assays, SW620 cells were co-transfected with GAL4-PPARα, GAL4-PPARγ or GAL4-c-Jun expression vector, together with a GAL4-LUC reporter plasmid. Upon transfection, complete medium with 0.5 % FBS was added to the cells and incubated at 37 °C for an additional 16 h. Transfected cells were exposed to different stimuli as indicated. Then, cells were harvested and lysed. Luciferase activity was determined by using the luciferase assay system (Promega) with a luminometer Monolight 2010 (Analytical Luminescence Laboratory). Protein measurements in extracts from transfected cells were performed with the BCA (bicinchoninic acid) protein assay (Pierce). The data presented are expressed as the means ± S.E.M. of the determinations in RLU (relative luciferase units) per μg of total protein in the cell extract or as fold induction (observed experimental RLU/basal RLU in the absence of any stimulus).

### EMSA (electrophoretic mobility-shift assay)

Nuclear extracts were prepared from SW620 cells as described previously [31]. Protein concentration was determined using the Bradford assay (Bio-Rad). Nuclear protein (5 μg) was incubated with 1 μg of poly(dI-dC) · (dI-dC) DNA carrier in DNA-binding buffer [2 % (w/v) poly(vinyl ethanol), 2.5 % (v/v) glycerol, 10 mM Tris/HCl, pH 8, 0.5 mM EDTA and 0.5 mM dithiothreitol] with 6 mM $MgCl_2$ for 10 min. The DNA-binding reactions was performed by adding 50 000 c.p.m. of $^{32}$P-labelled double-stranded AP-1 consensus oligonucleotide (5′-CGCTTGATGAG-TCAGCCGGAA-3′) (Promega) and incubated at room temperature (20 °C) for 15 min. A 30-fold molar excess of unlabelled oligonucleotide was added before the addition of the probe for competition when indicated. DNA–protein complexes were resolved by electrophoresis on 4 % non-denaturing polyacrylamide gels.

### Cell viability assay

The viability of the cells was measured by the use of the tetrazolium salt WST-1 (Roche Molecular Biochemicals) as described previously [31]. SW620 cells were incubated for 3, 6 and 24 h in the presence of increasing doses of PPARα ligands (10, 50 and 100 μM) and/or PMA + Ion and incubated with 10 % WST-1 reagent. The formazan dye produced by metabolically active cells was quantified by spectrophotometrical measurement.

### Statistical analysis

Data are expressed as means ± S.E.M. and their statistical significance was analysed using Student's $t$ test. $P < 0.05$ was considered to be significant. All the experiments shown are either representative or the mean of triplicate results of at least two independent experiments performed in order to guarantee the reproducibility and the significance of the results.

## RESULTS

### PPARα ligands LY-171883 and WY-14,643 inhibit phorbol-ester-induced COX-2 and VEGF expression in colon carcinoma cells

We first explored the influence of LY-171883 and WY-14,643 on gene expression in colon carcinoma cells by analysing the expression of COX-2 and VEGF in SW620 and in Caco-2 colon carcinoma cell lines. COX-2 and VEGF mRNA levels was determined by quantitative real-time RT–PCR analysis. These cells express low levels of COX-2 or VEGF mRNAs that increased

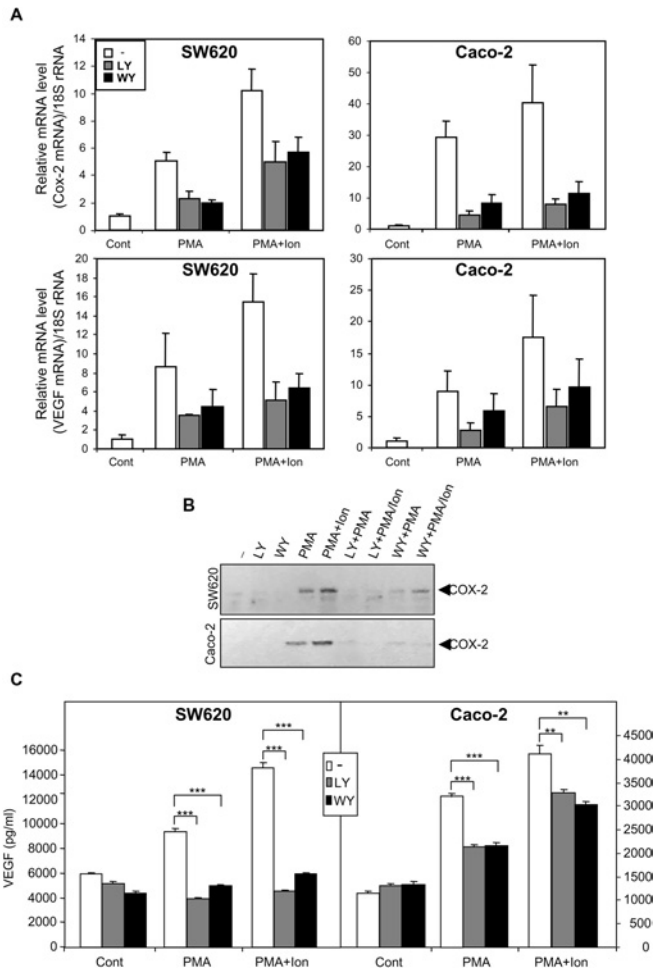


**Figure 1 PPARα ligands LY-171883 and WY-14,643 regulate COX-2 and VEGF expression**

LY-171883 (LY; 50 μM) or WY-14,643 (WY; 50 μM) was added to SW620 or Caco-2 cells 1 h with prior addition of PMA (15 ng/ml) or PMA + Ion (1 μM) as indicated. (**A**) Analysis of COX-2 and VEGF mRNA levels by quantitative real-time RT–PCR in cells treated with PMA or PMA + Ion for 16 h (SW620) or 6 h (Caco-2) in the presence or absence of PPARα ligands. (**B**) COX-2 protein levels were analysed by Western blot in extracts from cells treated with PMA or PMA + Ion for 16 h (SW620) or 6 h (Caco-2) in the presence or absence of LY-171883 or WY-14,643. (**C**) Production of VEGF in the supernatants of SW620 or Caco-2 cells after the different treatments was determined by ELISA. Results are means ± S.E.M. (**$P < 0.01$; ***$P < 0.001$).

upon treatment with PMA (15 ng/ml) or PMA plus Ion (1 μM). Addition of LY-171883 or WY-14,643 resulted in a strong inhibition of the induction of COX-2 and VEGF mRNA levels by PMA or PMA plus Ion (Figure 1A). Hence, we next analysed the effects of these PPARα ligands on COX-2 and VEGF protein expression in these cell lines. As shown in Figure 1(B), LY-171883 or WY-14,643 treatment substantially reduced the induction of COX-2 protein expression elicited by PMA or PMA + Ion stimulation. Phorbol ester treatment induced an increase in VEGF protein production in the supernatants of SW620 or Caco-2 cells that was significantly inhibited by pre-treatment with PPARα ligands (Figure 1C).

### PPARα signalling in SW620 colon carcinoma cells

LY-171883 and WY-14,643 are able to regulate gene expression by their ability to act as a PPARα activators [32]. Thus we analysed whether these drugs behaved as PPARα agonists in

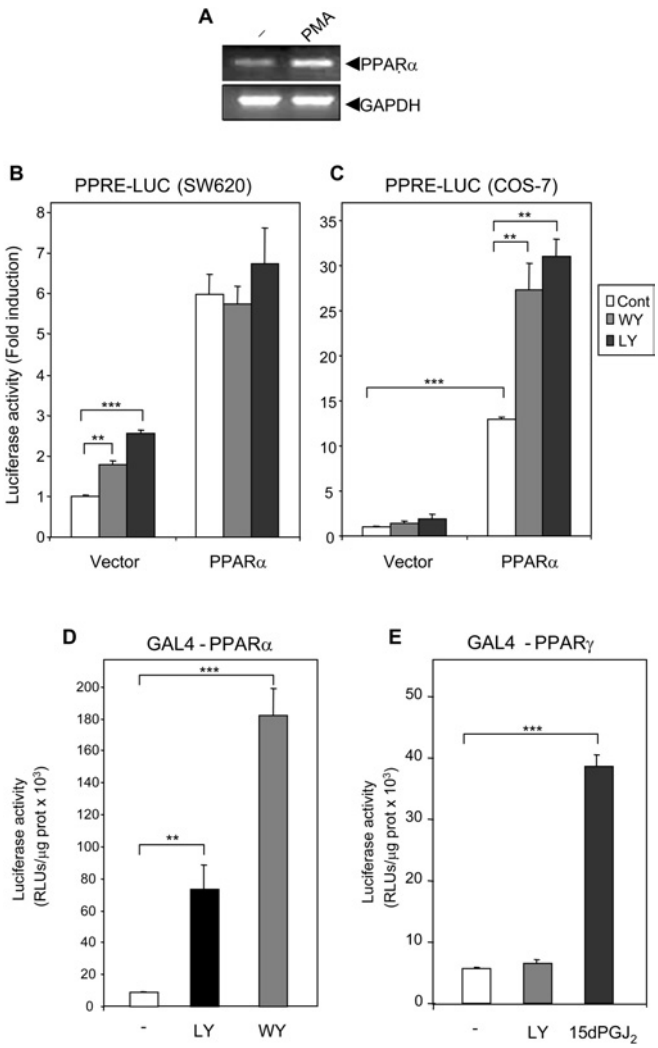


**Figure 2 LY-171883 and WY-14,643 mediate PPARα-dependent transcriptional activation in SW620 cells**

(**A**) RT–PCR analysis of PPARα mRNA expression in SW620 cells. An aliquot of the amplified DNA was separated on an agarose gel and stained with ethidium bromide for qualitative comparison. (**B**, **C**) Cells were cultured in the absence or presence of PMA (15 ng/ml) for 16 h. SW620 cells (**B**) or COS-7 cells (**C**) were transfected with the PPAR-responsive reporter plasmid PPRE-LUC along with a PPARα expression vector. After transfection, cells were treated for 16 h with LY-171883 (LY; 50 μM) or WY-14,643 (WY; 50 μM), and luciferase activity was determined. (**D**, **E**) SW620 cells were transiently transfected with a GAL4-LUC reporter plus expression vectors for the chimaeric construct GAL4-PPARα (**D**) or GAL4-PPARγ (**E**). Cells were incubated with PPARα agonists LY-171883 (LY; 50 μM) and WY-14,643 (WY; 50 μM) or with the PPARγ agonist 15d-$PGJ_2$ (1 μM) for 16 h. Results are means ± S.E.M. (**$P < 0.01$; ***$P < 0.001$).

SW620 cells. As shown in Figure 2(A), SW620 colon carcinoma cells express low levels of PPARα mRNA that were induced by PMA treatment. Moreover, LY-171883 and WY-14,643 were able to transactivate a PPRE-dependent luciferase reporter (PPRE-LUC), pointing to transcriptional regulation through endogenous PPARα (Figure 2B). Transcription driven by this PPRE was increased strongly upon co-transfection of a PPARα expression vector in SW620 cells, which could not be increased further by PPARα ligands. On the other hand, in COS-7 cells, which lack detectable amounts of endogenous PPARα (results not shown), PPAR agonists did not induce PPRE-driven transcription unless PPARα expression vector was co-transfected (Figure 2C). Both LY-171883 and WY-14,643 were able to induce ligand-dependent

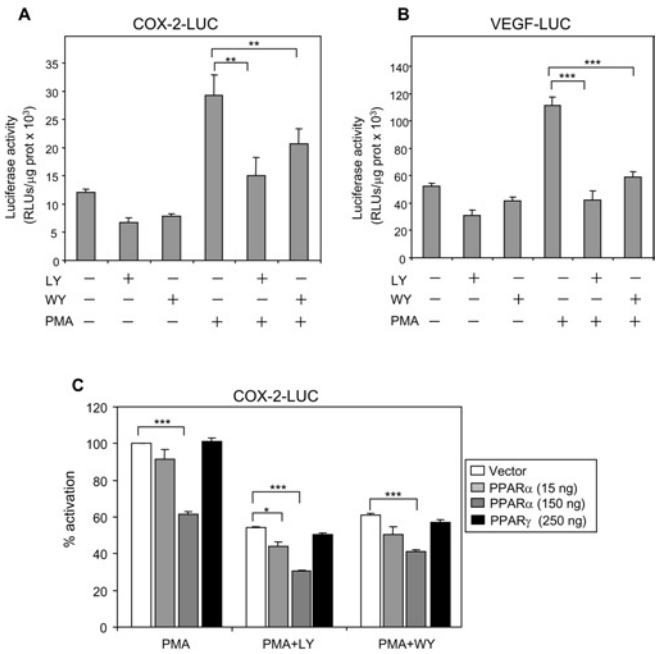


**Figure 3 Effects of PPARα agonists on COX-2 and VEGF promoter activity**

SW620 cells transfected with pCOX-2-LUC (**A**) or pVEGF-LUC (**B**) reporters were treated for 1 h with LY-171883 (LY; 50 μM) or WY-14,643 (WY; 50 μM), and then stimulated with PMA (15 ng/ml) for 16 h. Results are RLUs per μg of total protein in the cell extract (means ± S.E.M.). (**C**) SW620 cells were transiently transfected with the COX-2-LUC reporter along with an empty vector, or different quantities of PPARα or PPARγ expression plasmids as indicated. After transfection, cells were treated for 1 h with LY-171883 (LY; 50 μM) or WY-14,643 (WY; 50 μM), before PMA treatment. Cells were lysed, and luciferase activity was determined. Results are percentages (means ± S.E.M.) of activation by PMA considering the induction of promoter activity in the absence of treatment with PPAR vectors and ligands to be 100% (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

transactivation of a GAL4-PPARα construct, confirming further the ability of these drugs to act as PPARα ligands in SW620 cells (Figure 2D). Conversely, LY-171883 did not exert any effect in the transactivation mediated by a GAL4-PPARγ construct, which was efficiently induced by the PPARγ ligand 15d-$PGJ_2$ (Figure 2E). These results confirmed further that LY-171883 and WY-14,643 were able to positively regulate PPRE-driven transcription through binding to PPARα in the colon carcinoma cell line SW620.

### PPARα ligands inhibit transcriptional induction of VEGF and COX-2 promoters

We next analysed whether the effects of the PPARα ligands LY-171 883 and WY-14,643 on PMA-mediated induction of COX-2 and VEGF were taking place at the transcriptional level. In agreement with data obtained with the mRNA and protein, PMA strongly induced the transcription driven by human COX-2 (pCOX-2 LUC) or VEGF (pVEGF-LUC) promoters in SW620 cells (Figures 3A and 3B). Similarly to the effect observed on COX-2 and VEGF expression, pre-treatment with LY-171883 or WY-14,643 blunted PMA induction of COX-2 and VEGF promoters, reducing their activity to basal levels (Figures 3A and 3B).

As these results pointed to PPARα as a negative regulator of transcriptional induction of gene expression in these cells, we next explored the effect of increasing amounts of PPARα on PMA-mediated transcriptional induction of these genes. SW620 cells were transfected with increasing amounts of PPARα or PPARγ expression plasmids along with the COX-2 promoter construct.

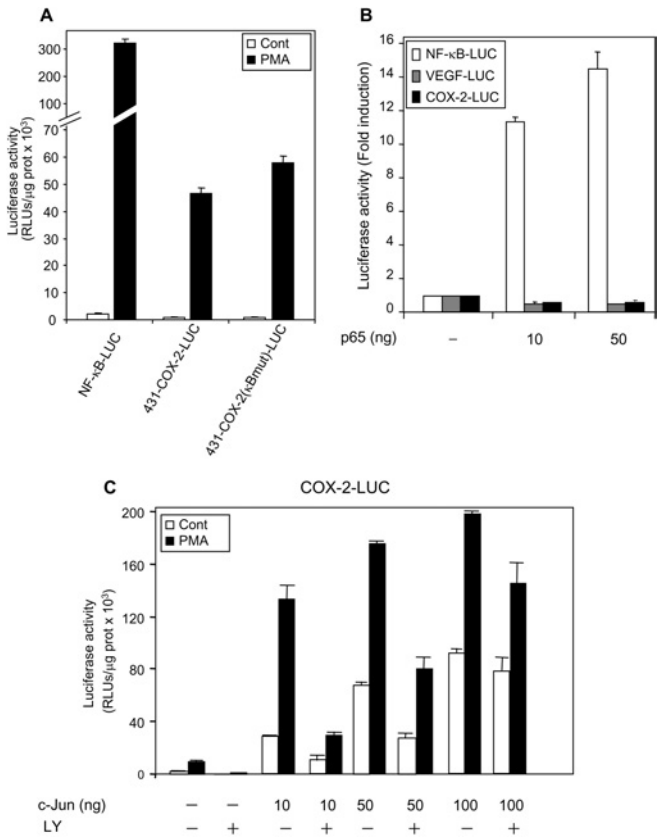


**Figure 4 PPARα ligands inhibit AP-1-mediated transcriptional induction of COX-2**

(**A**) SW620 cells were transfected with the NF-κB-LUC, 431-COX-2-LUC or 431-COX-2(κBmut)-LUC reporter genes in the presence or absence of PMA (15 ng/ml) for 16 h. (**B**) SW620 cells were co-transfected with the COX-2-LUC, VEGF-LUC and NF-κB-LUC reporter plasmids along with 10–50 ng of an expression vector for p65 NF-κB. (**C**) Cells were co-transfected with pCOX-2-LUC reporter plasmid along with 10–50 ng of the RSV-c-Jun expression plasmid. Cells were grown in the presence or absence of LY-171883 (LY; 50 μM) and stimulated with PMA (15 ng/ml) for 16 h. Results are means ± S.E.M.

As shown in Figure 3(C), PPARα expression inhibited in a dose-dependent manner the induction of COX-2 promoter activity by PMA. More interestingly, PPARα expression enhanced the inhibitory effects elicited by PPARα ligands. On the other hand, transfection of high amounts of a PPARγ expression vector did not show any significant effects in the absence or in the presence of LY-171883 or WY-14,643.

### Inhibition of AP-1-mediated transcriptional induction by PPARα agonists

It is well known that most of the inhibitory effects on gene transcription of PPARα agonists are due to their ability to inhibit various transcription factors such as NF-κB and AP-1 [16]. We determined the influence of AP-1 and NF-κB transcription factors on PMA-mediated transcriptional induction of COX-2 and VEGF in these cells as potential candidates to be affected by PPARα activation. As shown in Figure 4(A), PMA treatment of SW620 cells was able to activate NF-κB, increasing NF-κB-dependent transcription. However, mutation of the NF-κB-response element in the COX-2 promoter [431-COX-2(κBmut)-LUC] did not influence PMA-driven COX-2 transcriptional activation (Figure 4A). Furthermore, whereas overexpression of p65 NF-κB clearly induced NF-κB-LUC reporter activity, it did not affect the COX-2 or VEGF promoter. These results discarded an essential role of NF-κB in the regulation of COX-2 or VEGF expression upon PMA treatment in SW620 cells. Several reports have described the essential role of AP-1 activation in the regulation of COX-2 and VEGF gene expression in a variety of cell types, including colon carcinoma cells [33,34]. We have shown previously that overexpression of the AP-1 member c-Jun transactivated both COX-2 and VEGF promoters to a similar extent as the AP-1-driven −73Col-LUC reporter gene, thus confirming the involvement of this factor in the transcriptional induction of COX-2 and VEGF in SW620 cells [31]. Thus we next analysed the effect of PPARα agonists on c-Jun-mediated COX-2 promoter transcriptional induction. LY-171883 inhibited the c-Jun-mediated transactivation of COX-2 promoter, in both the presence and absence of PMA which co-operated with c-Jun overexpression to further enhance transcriptional activation (Figure 4). Noteworthily, expression of increasing amounts of a c-Jun expression plasmid were able to substantially reverse the effect elicited by LY-171-883, thus suggesting a negative cross-talk between c-Jun and PPARα signalling.

### Transcriptional interference between AP-1 and PPARα in the regulation of gene expression in colon carcinoma cells

It is well known that PPARα can affect transcriptional activation through interference with other transcription factors [6,12]. Interestingly, treatment of SW620 cells with LY-171883 or WY-14,643 inhibited the induction of the well-characterized AP-1-dependent −73Col-LUC reporter by PMA (Figure 5A). Co-transfection of increasing amounts of PPARα diminished further the induction of the AP-1 reporter construct elicited by PMA. On the other hand, co-transfection with higher doses of a PPARγ expression vector did not elicit any substantial effect in either the presence or absence of PPARα agonists. These data pointed to a negative cross-talk between AP-1 and PPARα signalling in the regulation of genes such as VEGF and COX-2 in colon carcinoma cells. In order to determine whether the transcriptional interference between PPARα and AP-1 activities occurs in a reciprocal manner, transfection assays were performed to test the effect of c-Jun on the PPARα-dependent activation of a PPRE-driven promoter. As expected, transfection with a PPARα expression vector consistently induced the PPRE reporter activity. Co-transfection of increasing amounts of c-Jun expression vector led to a dose-dependent inhibition of reporter activity induced by PPARα, without affecting basal promoter activity in the absence of co-transfected receptor (Figure 5B). These results confirm the existence of a mutual antagonism between c-Jun and PPARα signalling.

### PPARα ligands inhibit AP-1 activation at several levels

Once established that PPARα activation was inhibiting AP-1-dependent transcriptional activation, we next addressed the fine mechanism by which PPARα agonists was inhibiting AP-1-mediated responses. First, we tested the influence of LY-171883 or WY-14,643 treatment on AP-1 binding to DNA by EMSAs. As shown in Figure 6(A), a retarded protein complex that bound specifically to the AP-1 consensus sequence was observed in unstimulated cells. PMA treatment induced a strong increase in the formation of the retarded complex, which was severely impaired in cells pre-treated with LY-171883 or WY-14,643 before PMA stimulation. Transcriptional activation by c-Jun can be also modulated by the regulation of the activity of its intrinsic transactivation domain by serine phosphorylation [35]. As shown in Figure 6(B), the PPARα agonists LY-171883 and WY-14,643 significantly reduced PMA-induced transactivating

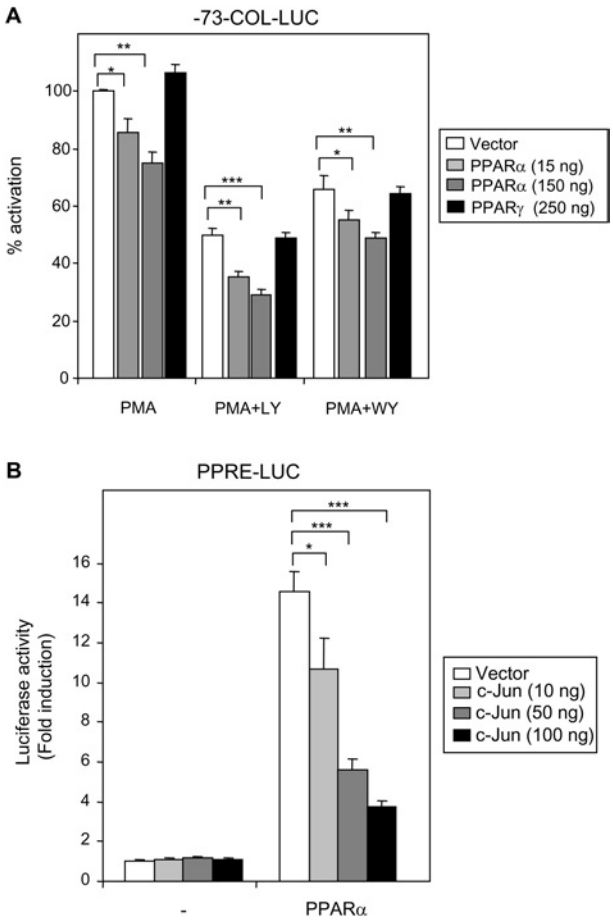


**Figure 5 Negative cross-talk between PPARα and AP-1**

(**A**) SW620 cells were transiently transfected with the AP-1 reporter construct — 73-Col-LUC together with different quantities of PPARα or PPARγ expression plasmids as indicated. After transfection, cells were treated for 1 h with LY-171883 (LY; 50 μM) or WY-14,643 (WY; 50 μM), before PMA treatment. Cells were lysed, and luciferase activity was determined. Results are percentages (means ± S.E.M.) of activation by PMA considering the induction of promoter activity in the absence of treatment with PPAR ligands to be 100 %. (**B**) SW620 cells were transiently transfected with the PPRE reporter construct PPRE-LUC along with increasing quantities of the c-Jun expression plasmid in the presence or absence of a PPARα expression plasmid. After transfection, cells were lysed, and luciferase activity was determined. Results are shown as fold induction over the observed RLUs in the absence of co-transfection of PPARα and c-Jun (means ± S.E.M.) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

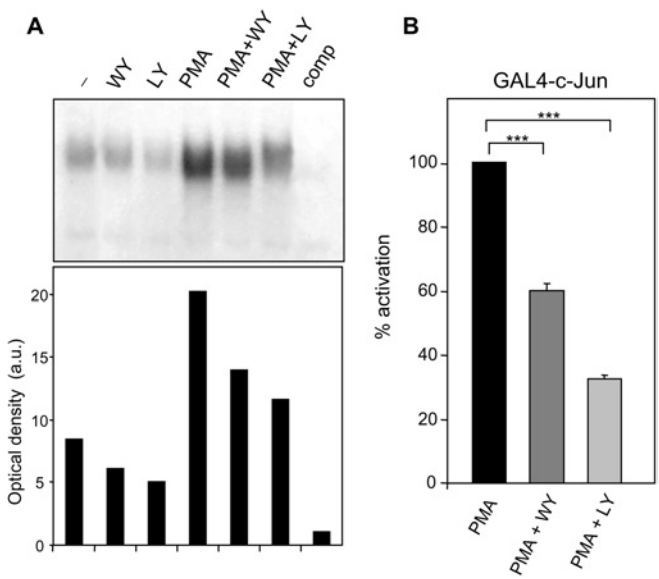


**Figure 6 PPARα ligands inhibit AP-1-mediated signalling**

(**A**) Nuclear extracts were obtained from cells incubated with LY-171883 (LY; 50 μM) or WY-14,643 (WY; 50 μM) for 2 h and then stimulated with PMA (15 ng/ml) for 4 h. Binding to a consensus labelled AP-1 probe was evaluated by EMSA. A 30-fold molar excess of unlabelled AP-1 consensus oligonucleotide was added to determine the specific binding (comp). The lower panel shows the densitometry of the radioactive bands in arbitrary units (a.u.). (**B**) Cells were transiently co-transfected with the reporter plasmid Gal4-LUC along with a GAL4-c-Jun expression vector. Cells were treated with PMA for 16 h in the absence or presence of LY-171883 (LY; 50 μM) or WY-14,643 (WY; 50 μM) and luciferase activity was determined. Results are represented as the percentage of activation by PMA considering 100 % the induction of reporter activity in the absence of treatment with PPAR ligands. Results are means ± S.E.M. (**$P < 0.01$; ***$P < 0.001$).

activity of the chimaeric protein GAL4-c-Jun, that contains the c-Jun transactivation domain (amino acids 1–166) fused to the DBD of the GAL4 yeast transcription factor.

## DISCUSSION

Previous studies have suggested an anti-inflammatory and anti-tumoural role of PPAR activators in a variety of experimental models (reviewed in [6,12,36]). Many of these effects are mediated by their ability to inhibit gene transcription of genes that are involved in inflammation, cell growth and angiogenesis [1,37,38]. In the present study, we have shown that PPARα agonists severely diminished phorbol-ester-mediated induction of VEGF and COX-2 expression in colon carcinoma cells. Accumulating evidence suggests a close relationship among inflammation, VEGF, COX-2, PPARs and cancer, particularly in the gastrointestinal tract. Chronic inflammation is a tumour promoter in almost all tissues and is implicated in the pathogenesis of several cancers, particularly those in the gastrointestinal tract. Indeed, patients with chronic inflammatory bowel diseases are at increased risk of developing colorectal cancer [39]. COX-2 and VEGF are crucial agents in inflammatory processes, cell proliferation and tumour growth, participating in promoting tumour-associated angiogenesis. These proteins are aberrantly expressed in colorectal carcinomas in comparison with normal intestinal epithelial cells and are associated with cell growth and tumour progression (reviewed in [18,19]). Epidemiological studies have demonstrated that NSAIDs, agents inhibiting COX-2-derived prostaglandin production, appear to be effective in cancer prevention [40]. Noticeably, some NSAIDs may act as PPARα and PPARγ ligands, suggesting that, in addition to inhibiting prostaglandin production, they might also regulate gene expression as part of their anti-inflammatory and chemopreventive mechanisms [41]. PPARs are expressed in the intestine at various levels, playing an important role in the development of colon carcinomas [11,42,43]. Although PPARγ is the predominant isoform, PPARα is also expressed in the colon, being able to participate in the differentiation of malignant tumour cells [8,44,45]. Several reports point to PPARα ligands as potentially beneficial chemopreventive agents in colon carcinogenesis. Tanaka and colleagues have demonstrated that PPAR agonists including bezafibrate, a PPARα ligand, are able to suppress chemically induced aberrant crypt foci formation in the rat colon [9,11]. In addition, this PPARα agonist has been reported to suppress intestinal polyp formation in $Apc^{-/-}$ (adenomatous polyposis coli) mice [10]. Moreover, methylclofenapate, a drug that displays properties as a PPARα agonist, is also able to reduce intestinal polyp size and number in $Apc^{-/+}$ mice [8]. Kohno et al. [9] have recently reported that both the COX-2 inhibitor nimesulide and PPAR ligands

inhibit colitis-related colon carcinogenesis. These authors also reported that bezafibrate, a PPARα agonist, significantly reduced the incidence of chemically induced colon adenocarcinoma in mice [9]. Interestingly, the suppressive effect of these drugs on the developing colonic adenocarcinoma correlated well with lowered expression of COX-2 in the colonic malignancies. Accordingly, the results of the present study suggest that PPARα activators may display antineoplastic effects by their ability to inhibit genes involved in colonic inflammation, such as COX-2 and VEGF, among others. This may prevent the development of aberrant crypt foci, thus acting before the first steps of carcinogenesis occur. Anti-inflammatory action of PPARα has been ascribed to the inhibition of genes that are involved in inflammation control in a variety of cell types [15,46]. In addition, our results show the PPARα-mediated inhibition of COX-2 and VEGF transcriptional activation in a colon carcinoma cell line.

Several molecular mechanisms have been proposed to explain the inhibitory actions of PPARα on gene transcription. Negative regulation of gene expression by PPARs might occur either by competition for limiting amounts of essential co-activators or through direct physical interactions between PPARs and specific transcription factors [1,7,47]. Our results demonstrate that PPARα-mediated inhibition of COX-2 and VEGF transcriptional activation occurs, at least in part, by interfering with AP-1-mediated activation. This interference is reciprocal, as expression of a transfected reporter gene linked to a PPRE was inhibited by co-transfection with a c-Jun expression plasmid. Accordingly, an excess of c-Jun was able to reverse the repressive effect of PPAR on AP-1-mediated COX-2 transcription. Our findings suggest that PPARα interference on PMA-induced COX-2 and VEGF transcription in colon carcinoma cells occurs primarily through AP-1, discarding the involvement of NF-κB in the regulation of these genes by PMA in the colon carcinoma cell line SW620. In this sense, Staels et al. [15] have reported the inhibition of COX-2 transcriptional activation by interleukin 1 as a result of PPARα repression of NF-κB signalling in human aortic smooth-muscle cells. Negative cross-talk between PPARs and AP-1 has been described extensively. PPARs are able to inhibit the ability of c-Jun to activate transcription of endothelin-1 [48,49]. Conversely, c-Jun inhibits the ability of PPARs to activate PPRE-driven genes [16]. Direct interaction between PPARα and transcription factors has been identified as a mechanism for PPARα-mediated repression of gene expression. Thus previous studies have shown a direct interaction of PPARα with c-Jun [16]. Accordingly, our results are compatible with a direct interaction between c-Jun and PPAR. This interaction may explain the decrease in AP-1 binding to DNA found after PPARα agonist treatment in stimulated SW620 cells. In addition to the interference on AP-1 binding to DNA, our results demonstrate that inhibition of AP-1-dependent activity by PPARα activators may also occur by diminishing the intrinsic c-Jun transactivating activity. AP-1 activity is dependent on the transcriptional and post-transcriptional activation of its components, members of Fos and Jun families [50]. c-Jun is considered to be the main component of AP-1 and its activity is regulated post-transcriptionally by JNK (c-Jun N-terminal kinase) phosphorylation at $Ser^{63}$ and $Ser^{73}$ on its transactivating domain [50]. Interestingly, PPARα is able to interact with the JNK-responsive part of c-Jun [16] and PPARα activators have been shown to be able to inhibit c-Jun phosphorylation by JNK [49]. PPARα-mediated interference on c-Jun-mediated transactivation may thus occur through interference with the phosphorylation of c-Jun in its transactivation domain that might also alter its association with critical co-activators that are necessary for transcriptional activation. Nevertheless, additional studies are necessary to define the precise molecular mechanisms that are involved in the negative regulation of AP-1 activation by PPARα in colon carcinoma cells.

Taken together, the results of the present study and those findings of previous studies suggest that PPARα activation may be beneficial in the early stages of colon tumorigenesis through inhibition of AP-1-mediated transcriptional activation of genes involved in inflammation such as COX-2 and VEGF. Inhibition of colonic inflammation by PPARα ligands might be responsible for their potential chemopreventive effects on inflammation-associated colon carcinogenesis. However, it must be taken into account that many of the reported effects of PPAR ligands *in vitro* await confirmation by additional basic and clinical research to ascertain whether they can be considered of pharmacological significance *in vivo* in humans.

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